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Characterization of Biotinylated Repair Regions in Reversibly Permeabilized Human Fibroblasts†

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ABSTRACT: We have examined the incorporation of biotinyl-11-deoxyuridine triphosphate (BiodUTP) into excision repair patches of UV-irradiated confluent human fibroblasts. Cells were reversibly permeabilized to BiodUTP with lysolecithin, and biotin was detected in DNA on nylon filters using a streptavidin/alkaline phosphatase colorimetric assay. Following a UV dose of 12 J/m^2, maximum incorporation of BiodUTP occurred at a lysolecithin concentration (80–100 μg/mL) similar to that for incorporation of dTTP. Incorporation of BiodUTP into repair patches increased with UV dose up to 4 and 8 J/m^2 in two normal human fibroblast strains, while no incorporation of BiodUTP was observed in xeroderma pigmentosum (group A) human fibroblasts. The repair-incorporated biotin was not removed from the DNA over a 48-h period, and only slowly disappeared after longer times (~30% in 72 h), while little of the biotin remained in cells induced to divide. Furthermore, the stability of the biotin in repaired DNA was unaffected by a second dose of UV radiation several hours after the biotin-labeling period to induce a "second round" of excision repair. Exonuclease III digestion and gap-filling with DNA polymerase I indicate that the majority of biotin-labeled repair patches (~80%) are rapidly ligated in confluent human cells. However, the remaining patches were not ligated after a 24-h chase period, in contrast to dTTP-labeled repair patches. The BiodUMP repair label in both chromatin and DNA is preferentially digested by staphylococcal nuclease, preventing the use of this enzyme for nucleosome mapping in these regions. However, restriction enzyme and DNase I digestions of the isolated nuclei demonstrate that at least some of the repair-incorporated BiodUMP becomes associated with nucleosome core DNA following nucleosome rearrangement. Therefore, the biotin tag does not appear to prevent the folding of nascent repair patches into native nucleosome structures.

The biotinylated deoxyuridine triphosphate nucleotide (BiodUTP)[1] developed by Langer et al. (1981) has been used extensively to label DNA. Incorporation of biotinylated nucleotides into DNA has been accomplished enzymatically in vitro (Langer et al., 1981), as well as in vivo (Hiriyanna et al., 1988). By exploiting the strong interaction ($K_d = 10^{-15}$) between biotin and streptavidin, a 60-kDa protein isolated from the bacterium *Streptomyces avidinii*, picogram quantities of biotinylated DNA can be detected when streptavidin is coupled to an appropriate indicator molecule such as alkaline phosphatase (Leary et al., 1983). The extremely sensitive streptavidin–biotin affinity system has been used to isolate DNA–protein complexes (LeBlond-Francillard et al., 1987; Vincenz et al., 1991), and was employed to visualize replicated DNA in the electron microscope (Hiriyanna et al., 1988).

Hunting et al. (1985a) have shown that BiodUTP is also incorporated into DNA during repair synthesis in mechanically disrupted human cells. Mammalian cells are not permeable to highly charged molecules, including nucleotide triphosphates, and therefore require permeabilization prior to BiodUTP addition. However, in the studies by Hunting et al. (1985a), cells are irreversibly permeabilized by mechanical disruption, and this method does not allow examination of repaired regions at long times after biotin labeling. Transient permeabilization of cell membranes to small molecules is possible, however, with lysolecithin under conditions where the cells remain viable (Castellot, 1980). Indeed, Lorenz et al. (1988) demonstrated that confluent human fibroblasts exposed

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[1] Abbreviations: dNTPs, 2′-deoxynucleotide 5′-triphosphates; BiodUTP, 5-[*N*-(*N*-biotinyl-ε-aminocaproyl)-3-aminoallyl]-2′-deoxyuridine 5′-triphosphate; BiodUMP, 5-[*N*-(*N*-biotinyl-ε-aminocaproyl)-3-aminoallyl]-2′-deoxyuridine 5′-monophosphate; dThd, thymidine; PBS, phosphate-buffered saline; UV, ultraviolet; dCyd, deoxycytidine; dTTP, thymidine 5′-triphosphate; dTMP, thymidine 5′-monophosphate; dCTP, 2′-deoxycytidine 5′-triphosphate; dCMP, 2′-deoxycytidine 5′-monophosphate.

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to a physiological dose of UV radiation, and reversibly permeabilized to dNTPs by lysolecithin, can be used to study long-term events in newly repaired regions in chromatin. Following this permeabilization and repair of most UV photoproducts, these cells remain viable and have the same survival as unpermeabilized cells (Lorenz et al., 1988).

In order to use biotinylated nucleotides to isolate newly repaired chromatin, it is necessary to demonstrate that this nucleotide analogue does not significantly alter the excision repair process, and is not removed in vivo during (or after) repair synthesis. Therefore, we have examined (a) the incorporation of BiodUTP into DNA of confluent human fibroblasts which were exposed to UV radiation prior to permeabilization with lysolecithin, (b) the stability of biotinylated repair patches in intact cells many hours after incorporation, and (c) the completion of the newly repaired regions containing BiodUMP.

Materials and Methods

Cell Culture. Human diploid fibroblasts (strains AG1518 and IMR 90; Human Genetic Mutant Cell Repository, Camden, NJ) were grown in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum and 5% newborn calf serum, as described by Smerdon et al. (1982). Xeroderma pigmentosum human fibroblasts, complementation group A (strain XP12BE; American Type Culture Collection, Rockville, MD), were grown in the same medium not containing newborn calf serum, but containing 10% fetal bovine serum. In some experiments, the cells were labeled with 5–25 nCi/mL [^{14}C]dThd (40–60 mCi/mmol, New England Nuclear, Wilmington, DE) for 1 week starting 24 h after the cells were split 1:3. The medium was replaced with fresh medium, and the cells were allowed to grow to confluence (3 weeks).

UV Irradiation and Repair Synthesis. Confluent cells were UV-irradiated and were permeabilized with lysolecithin (Sigma, St. Louis, MO; type I) as previously described (Lorenz et al., 1988). Briefly, after the cells were rinsed twice with ice-cold modified PBS buffer (3 mM NaH_2PO_4, 13 mM Na_2HPO_4, pH 7.2, 135 mM NaCl, 5 mM KCl, and 1 mM $CaCl_2$), they were irradiated with various doses of UV light (2 W/m^2, predominantly 254 nm). The cells were then placed on ice and incubated with various concentrations of lysolecithin dissolved in modified PBS for 2 min. Following permeabilization, the lysolecithin was replaced with a repair reaction mix (35 mM HEPES, pH 7.4, 50 mM sucrose, 80 mM KCl, 5 mM $MgCl_2$, 7.5 mM KH_2PO_4, 1 mM $CaCl_2$, 5 mM ATP, 3 μM dATP, 3 μM dGTP, 3 μM dCTP, and 3 μM dTTP). Depending on the experiment, 3 μM BiodUTP [Biotin-11-dUTP; Bethesda Research Laboratories, Gaithersburg, MD; or synthesized according to the method of Langer et al. (1981)] was added instead of dTTP. In some experiments, dCTP was replaced with 20 μCi/mL [^{3}H]dCTP (16–20 Ci/mmol; ICN Biomedicals, Irvine, CA), or 20 μCi/mL [^{3}H]dTTP (47 Ci/mmol; Amersham, Arlington Heights, IL) was added instead of dTTP. The cells were incubated for varying times at 37 °C, and were harvested immediately, or the repair mix was replaced with conditioned medium containing 50 μM dTTP and 50 μM dThd. In addition, if [^{3}H]dCTP had been present in the repair mix, 30 μM dCTP and 30 μM dCyd were added, and the incubation was continued for several time periods. The cells were harvested and nuclei were isolated as described by Smerdon et al. (1979). The DNA was isolated by incubating nuclei with 200 μg/mL proteinase K (Boehringer Mannheim, Indianapolis, IN) and 1% SDS overnight at 37 °C followed by ethanol precipitation. Recently, it has been reported (Larone & Hunting, 1991) that proteinase K used during the isolation of biotinylated DNA may cause the loss of biotin via cleavage at the amide bond in the linker between dUTP and biotin. We have tested this by incubating biotinylated plasmid DNA fragments (BioVentures, Murfreesboro, TN) or biotinylated λ DNA (Bethesda Research Laboratories) with or without 200 μg/mL proteinase K for 6 h at 37 °C. After visualization and quantitation of the biotin remaining, we found no difference in the amount of biotin in both preparations (Table I).

Table I: Stability of Biotin in DNA Treated with or without Proteinase K[a]

DNA concn (ng)	detection (%)[b] −proteinase K	+proteinase K
1	23	25
2	43	47
3	48	48
4	61	61
6	66	64
8	79	69
10	91	77
12	88	97
14	95	96
16	97	99
18	100	99

[a] Biotinylated plasmid DNA was incubated at 37 °C with or without proteinase K (200 μg/mL) for 6 h, and the DNA was transferred to a nylon membrane via a slot blot apparatus. [b] The biotin was visualized with a streptavidin/alkaline phosphatase assay using 5-bromo-4-chloro-3-indolyl phosphate (BCP) and nitroblue tetrazolium (NBT) as substrates (Materials and Methods). The amount of incorporation was determined from a laser densitometer scan of the membrane (e.g., see Figure 1). The resultant areas of the absorbance peaks were normalized to the area of the absorbance peak of the highest concentration of DNA that had not been treated with proteinase K.

Measurement of Repair Synthesis. Radioactivity in isolated DNA labeled during repair synthesis with [^{3}H]dCTP or [^{3}H]dTTP was measured in a Beckman LS 7500 liquid scintillation counter. DNA that had been labeled with BiodUTP was blotted on nylon membranes (Cuno, Inc., Meriden, CT) using a Hoefer SlotBlot 24 well filtration manifold. Incorporation of BiodUTP was visually detected with the BluGENE nonradioactive nucleic acid detection system (Bethesda Research Laboratories) or by the method developed by Richterich et al. (1989). Membranes were scanned on a laser densitometer (LKB Model 2222), and the net absorbance was then divided by the amount of DNA that was blotted, determined from absorbance scans of the individual samples or by the ^{14}C DPMs of the individual samples if the DNA had been prelabeled.

Exonuclease III Digestions. DNA from cells prelabeled with [^{14}C]dThd and labeled during repair synthesis with BiodUTP and [^{3}H]dCTP (or [^{3}H]dTTP) was digested with *Escherichia coli* exonuclease III (Bethesda Research Laboratories) as described by Smerdon (1986). The samples were assayed for acid-soluble radioactivity as described by Smerdon et al. (1978). Aliquots that had been labeled with BiodUTP and [^{3}H]dCTP during repair synthesis were divided into two samples. The acid-soluble radioactivity of one of the samples was determined while the biotin remaining in the DNA in the other sample was visualized on slot blots.

E. coli DNA Polymerase I Assay. Confluent human AG1518 cells, prelabeled with [^{14}C]dThd, were damaged as above and incubated in repair mix with or without dNTPs for 30 min. BiodUTP was substituted for dTTP. The cells were harvested immediately or were chased for 24 h as described above. The isolated DNA was dissolved in DNA polymerase I buffer (50 mM Tris-HCl, pH 7.8, 5 mM $MgCl_2$, 10 mM

β-mercaptoethanol, 10 μg/mL BSA, 10 μM each of all four dNTPs, and 1 μCi of [^{3}H]dCTP/μg of DNA) (Maniatis et al., 1982). *E. coli* DNA polymerase I (Boehringer Mannheim) was added (5 units/μg of DNA), and the samples were incubated for 60 min at 15 °C. The unincorporated nucleotides were removed with a Prep-a-Gene DNA purification kit (Bio-Rad, Richmond, CA). The ^{3}H and ^{14}C DPMs were used to determine the percent of unligated repair patches that were present in the DNA.

Staphylococcal Nuclease Digestions. Nuclei from cells prelabeled with [^{14}C]dThd and labeled during repair synthesis with BiodUTP and [^{3}H]dCTP for 30 min, and then chased for 0 or 24 h, were suspended by Dounce homogenization in 10 mM Tris-HCl, pH 8.0, 0.25 M sucrose, and 0.1 mM $CaCl_2$. The nuclei were digested with staphylococcal nuclease (Worthington, Freehold, NJ) at a concentration of 0.1 unit/μg of DNA according to the method of Smerdon et al. (1978). Aliquots were removed at various times and were assayed for acid-soluble radioactivity and biotin content.

Restriction Enzyme Digestions. Nuclei from cells repair-labeled with BiodUTP for 2 h were isolated as described above. Nuclei were suspended in 10 mM Tris-HCl, pH 8.0, 50 mM NaCl, and 3 mM $MgCl_2$. The nuclei were digested with *Hae*III and *Rsa*I (Boehringer Mannheim), each at a concentration of 5 units/μg of DNA, for 3 h at 37 °C. Another aliquot of nuclei was resuspended in 10 mM Tris-HCl, pH 8.0, 0.25 M sucrose, and 0.1 M $CaCl_2$, and digested with staphylococcal nuclease (0.2 unit/μg of DNA) for 5 min at 37 °C. The isolated DNA was subjected to electrophoresis on a 1.5% agarose gel. The DNA was transferred to a nylon membrane, and the incorporated biotinylated nucleotides were visualized as described above.

DNase I Digestions. Nuclei from cells labeled with BiodUTP during the repair reaction for 30 min, and then chased for 0 or 24 h, were incubated at 37 °C with DNase I (0.01 μg/μg of DNA; Sigma) in 10 mM Tris-HCl, pH 8.0, 0.25 M sucrose, and 0.1 mM $CaCl_2$ for 30 min (Smerdon & Lieberman, 1980). Electrophoresis of the isolated DNA on a 7 M urea and 8% polyacrylamide gel was carried out according to Maniatis et al. (1982). The DNA was transferred to a nylon filter by electroblotting. The incorporated biotinylated nucleotides were visualized as described earlier.

Results

Labeling Excision Repair Patches with BiodUTP. Incorporation of BiodUTP into excision repair patches was detected in isolated DNA with a strepatvidin/alkaline phosphatase colorimetric reaction. An example of the data obtained with this system is shown in Figure 1A. The DNA was transferred to a nylon filter via a slot blot manifold, and the biotin was visualized (Materials and Methods). The amount of incorporation was quantiated by scanning the slot blots on a laser densitometer and normalizing the absorbance signal to the amount of DNA loaded (Figure 1B). We found that the absorbance between 0 and 1.1 (above background) increased linearly with increasing concentrations of biotinylated λ DNA in which 10–30 biotinylated nucleotides per kilobase had been substituted (data not shown). Since only a small fraction of the total DNA is associated with biotin-containing repair patches after a physiological dose of UV light, saturation of the biotin detection system is unlikely.

We first determined the concentration of lysolecithin at which maximal incorporation of BiodUTP occurred during repair synthesis in two strains (IMR 90 and AG1518) of confluent human fibroblasts. Cells were damaged with a UV dose of 12 J/m^2 and then permeabilized with various concentrations of lysolecithin. After 2 min, the lysolecithin was removed, and the cells were incubated for 2 h in a repair synthesis reaction mixture which included BiodUTP (Materials and Methods). Following the incubation, the DNA was isolated, and the incorporation of BioUTP was visualized and quantitated (Figure 2). Maximal incorporation of BiodUTP occurred at 80 μg/mL lysolecithin for AG1518 cells, and at 100 μg/mL for the IMR 90 strain. Little incorporation was seen in the unirradiated permeabilized cells (Figure 2, open symbols), indicating that little (or no) DNA replication took place. These results closely resemble the results obtained by Lorenz et al. (1988) when [^{3}H]dTTP was used as the repair label.

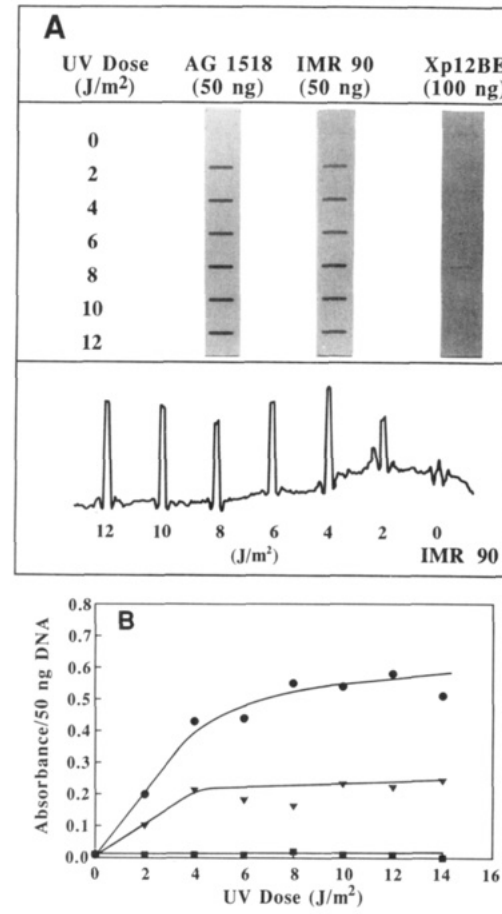

FIGURE 1: (A) Detection of repair-incorporated BiodUMP. Confluent human fibroblasts were irradiated with different doses of UV light and permeabilized with 80 μg/mL lysolecithin as described by Lorenz et al. (1988). The cells were incubated at 37 °C for 2 h in DNA repair mix containing 3 μM BiodUTP (Materials and Methods). DNA was isolated and blotted onto a nylon membrane. The BiodUMP incorporated was visualized as described in Table I. The upper panel shows a photograph of slot blot lanes for different human fibroblast strains irradiated at the indicated UV doses. The lower panel shows the resulting laser densitometer scan for one of these lanes (IMR 90 cells). (B) Results of quantitation of repair synthesis in permeabilized normal human fibroblasts [strains AG1518 (●) and IMR 90 (▼)] and xeroderma pigmentosum (group A) human cells [strain XP12BE (■)] following irradiation with different UV doses. The amount of incorporation is given as the peak absorbance from the laser densitometer scan of slot blots [shown in (A)] divided by the amount of DNA transferred to the membrane (Materials and Methods).

We also examined the dependence of repair synthesis on UV dose. For these experiments, three strains of human fibroblasts were grown to confluence and irradiated with various doses of UV light, permeabilized with 80 μg/mL lysolecithin, and repair-labeled with BiodUTP for 2 h. Incorporation of repair patches was dependent on both the UV dose and the human fibroblast strain used (Figure 1B). One of the normal human

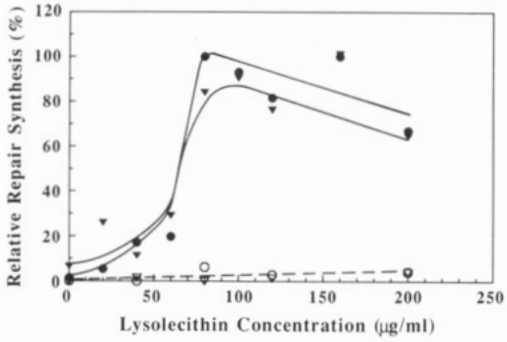

FIGURE 2: Biotin-labeled repair synthesis in UV-irradiated human fibroblasts as a function of lysolecithin concentration. Normal human fibroblasts [strains AG1518 (●) and IMR 90 (▼)] were irradiated with 12 J/m^2 UV light, permeabilized with various concentrations of lysolecithin, and incubated at 37 °C with 3 μM each of BiodUTP, dATP, dGTP, and dCTP for 2 h. The DNA was isolated, and the incorporated BiodUMP was determined as described in Figure 1. The open symbols show the values obtained for AG1518 (○) and IMR 90 (▽) cells that were treated as described above except the irradiation step was omitted. All values were normalized to the value for irradiated AG1518 cells at a lysolecithin concentration of 80 μg/mL.

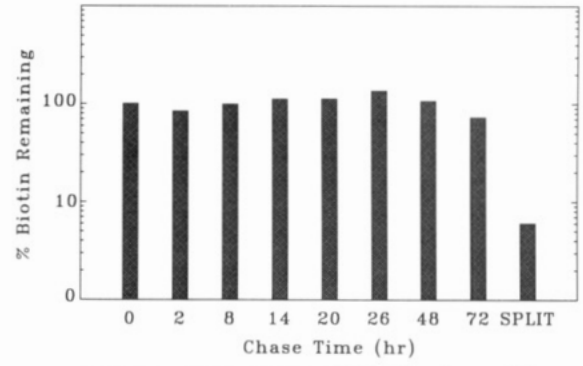

FIGURE 3: Stability of BiodUMP in DNA following excision repair synthesis. Normal human fibroblasts (strain AG1518) were irradiated with 12 J/m^2 UV light, permeabilized with 80 μg/mL lysolecithin, and incubated for 2 h at 37 °C with 3 μM BiodUTP in DNA repair mix. Cells were harvested immediately or incubated with conditioned medium containing 50 μM dThd and 50 μM dTTP for various times prior to harvest. Some of these cells were split 1:2 after 72 h of repair incubation and incubated an additional 48 h. In each case, DNA was isolated, and the BiodUMP incorporated was determined as described in Figure 1. Values are percent of the 0-h time point.

fibroblast strains (AG1518) yielded saturation in repair synthesis at about 8 J/m^2 while the other strain (IMR 90) yielded saturation at ~4 J/m^2. These values are similar to those for intact AG1518 (Dresler & Lieberman, 1983a) and IMR 90 cells (Smerdon & Lieberman, 1978a) when [^{3}H]dThd was used as the repair label. On the other hand, no incorporation of BiodUTP was observed in the repair-deficient xeroderma pigmentosum cells (complementation group A) (Figure 1B). Furthermore, the difference in overall repair incorporation between the two normal cell strains is also observed in intact cells repair-labeled with [^{3}H]dThd (unpublished results). These results strongly suggest that BiodUTP is inserted into nascent excision repair patches of lysolecithin-permeabilized cells.

Stability of BiodUMP in Repaired Regions. Since we are interested in studying features of newly repaired chromatin following long time periods, it is important to determine if BiodUMP is removed from labeled repair patches in intact cells. To investigate this possibility, AG1518 cells were damaged, permeabilized, and repair-labeled with BiodUTP for 2 h as above. Some cells were harvested immediately following the repair labeling period while in other cells the BiodUTP was chased with dTTP and dThd for various times. [dThd was included to "optimize" the effectiveness of the chase since >80% of the cells "reseal" their membranes within the 2-h BiodUTP labeling period (Lorenz et al., 1988).] The biotin-tagged repair patches that were inserted immediately after irradiation were not removed from the DNA over a 48-h period (Figure 3). After longer times, the biotin slowly disappeared from these regions (e.g., ~30% in 72 h) if the cells remained confluent. However, when cells were split after a 72-h chase period to stimulate cell division, the amount of biotin-labeled DNA was substantially reduced after 48 h (or following one or two rounds of replication) (Figure 3). From the value measured (6% remaining), between 76% and 88% of the biotin label disappeared from the DNA, if one corrects for the unbiotinylated DNA added during replication. This could have resulted, in part, from removal of BiodUMP during replicative synthesis, but at least some of this disappearance was caused by increased cell death (data not shown).

We also examined the effect of initiating a "second round" of excision repair on the BiodUMP stability, since excision repair may be processive and may be "reinitiated" to scan *previously repaired* DNA. For these experiments, cells were irradiated with 12 J/m^2 UV light, permeabilized, and pulse-labeled with BiodUTP for 3 h as before. At this time, some of the cells were irradiated a second time with 12 J/m^2 UV light to induce a second round of excision repair. The cells were incubated for 3 more h without addition of a labeled nucleotide. Since the "fast phase" of excision repair in AG1518 cells is not completed until 16–24 h after irradiation (Jensen & Smerdon, 1990), at which time some 70% of the pyrimidine dimers have been removed, we also examined BiodUMP removal during the "slow phase" of repair. In this case, cells were incubated for 24 h in chase medium before they were given a second dose of UV light. These cells were then incubated for an additional 24 h. The results indicate that following reinitiation of either the "fast phase" (3 h) or the "slow phase" (24 h) of excision repair, BiodUMP was not removed from the previously repaired DNA of confluent cells (Table II).

Table II: Stability of BiodUMP in Repaired Regions after a Second UV Dose[a]

time (h) after first dose	second UV dose (12 J/m^2)	time (h) after second dose	% biotin remaining
0	−	3	100
0	+	3	93
24	−	24	98
24	+	24	97

[a] Normal human fibroblasts (strain AG1518) were irradiated with 12 J/m^2 UV light, permeabilized with 80 μg/mL lysolecithin, and incubated at 37 °C with 3 μM each of BiodUTP, dATP, dGTP, and dCTP for 3 h. Some of the cells were then incubated with conditioned medium containing 50 μM dThd and 50 μM dTTP for 24 h. A second set of cells was irradiated with another dose of 12 J/m^2 UV light, and incubated for an additional 3 or 24 h. The DNA was isolated, and the amount of incorporated BiodUMP was determined as described in Figure 1.

Ligation of BiodUMP-Containing Repair Patches. We also examined ligation of the biotin-labeled repaired regions. For these experiments, confluent AG1518 cells were prelabeled with [^{14}C]dThd, irradiated with 12 J/m^2 UV light, and permeabilized as described earlier. The cells were then repair-labeled for 30 min in repair mix containing either [^{3}H]dTTP, or [^{3}H]dCTP and BiodUTP. The cells were harvested immediately, or incubated in chase medium for 24 h, and the DNA was digested with *E. coli* exonuclease III, as described by Smerdon (1986). The 3′→5′ exonuclease activity of exonuclease III rapidly digests unligated repair patches to acid-

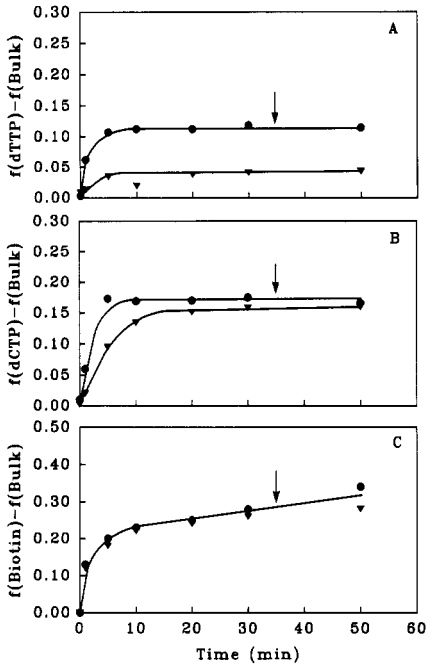

FIGURE 4: Difference in the fraction of repair-incorporated label and bulk DNA label released as a function of time of digestion with exonuclease III. Normal human fibroblasts (AG1518 cells), prelabeled with [^{14}C]dThd, were permeabilized with 80 μg/mL lysolecithin and labeled with [^{3}H]dTTP (A) or both [^{3}H]dCTP (B) and BiodUTP (C) for 30 min after 12 J/m^2 UV irradiation. The cells were harvested immediately (●) or incubated for 24 h in conditioned medium containing 50 μM dThd, 30 μM dCyd, 50 μM dTTP, and 30 μM dCTP (▼). The isolated DNA was incubated for various times at 37 °C with *E. coli* exonuclease III (0.5 unit/μg of DNA), and the acid-soluble radioactivity was determined as described by Smerdon (1986). After 35 min, the exonuclease III concentration was increased to 1.3 units/μg of DNA (arrow), and the incubation was continued for an additional 15 min. Values for biotin-labeled DNA were determined from the signal obtained on slot blots after each repair time (Figure 1). Values represent the difference between the fraction of repair label (^{3}H or biotin) digested and the fraction of bulk label (^{14}C) digested as a function of time (Smerdon, 1986).

soluble form, while ligated patches are digested at the same rate as the rest of the genomic DNA (Hunting et al., 1985b; Smerdon, 1986). Therefore, the extent of ligation is determined from the fraction of repair-incorporated label that is not rapidly released by exonuclease III into acid-soluble form.

As shown in Figure 4A, ~12% of the [^{3}H]dTTP-labeled repair patches are not ligated following a 30-min pulse. This result is similar to that observed by Lorenz et al. (1988) for lysolecithin-permeabilized cells. After a 24-h chase period, ~4% of the patches remain unligated by this assay (Figure 4A). In order to examine ligation of biotin-containing repair patches, the fraction of biotin remaining (per unit DNA) after exonuclease III digestion was determined from the slot blot analysis described in Figure 1 (also see Materials and Methods). To compare the different methods used to detect the fraction of repair label that was digested, DNA was labeled with both BiodUTP and [^{3}H]dCTP. The difference of the acid-soluble fraction of ^{3}H and ^{14}C was compared with the difference of the fraction of "biotin lost" and ^{14}C. These exonuclease III digestions indicated that the majority of the biotin-labeled repair patches were also efficiently ligated. After the 30-min repair-labeling period, ~17% of the [^{3}H]dCMP and ~22% of the biotin label are found in the "rapidly digested" fraction (Figure 4B,C). However, no further ligation occurred with either the [^{3}H]dCTP or the BiodUTP labels after a 24-h chase period, in contrast to the [^{3}H]dTMP-labeled repair patches (Figure 4).

Table III: Fraction of Repair Sites Extended by DNA Polymerase I[a]

UV dose 12 J/m^2	dNTPs	chase time (h) after UV dose	^{3}H/^{14}C ratio	%
−	+	0	0.66	0
+	−	0	1.62	100
+	+	0	0.88	23
+	+	24	0.89	24

[a] Human fibroblasts, prelabeled with [^{14}C]dThd, were irradiated with 12 J/m^2 UV light, permeabilized with 80 μg/mL lysolecithin, and incubated at 37 °C with or without dNTPs (BiodUTP was substituted for dTTP). The cells were harvested immediately after the repair labeling time, or chased for 24 h. The amount of incorporation of [^{3}H]dCTP (by DNA polymerase I) and [^{14}C]dThd in the isolated DNA was determined (Materials and Methods), and the ratio of ^{3}H DPM/^{14}C DPM is shown.

It is possible that the exonuclease III assay yields a misrepresentation of the fraction of unligated biotinylated repair patches. For example, a biotinylated base may block further digestion by the exonuclease. Therefore, we also tested ligation efficiency using an assay which involves filling in gaps at incomplete repair patches using DNA polymerase I. In this case, confluent prelabeled cells were damaged with UV light, permeabilized, and incubated with or without nucleotides (including BiodUTP) during repair synthesis. In lysolecithin-permeabilized cells, the endogenous dNTP pools are very low (Lorenz et al., 1988), and DNA repair synthesis is dependent on exogenous dNTPs. In the absence of dNTPs, only the first steps in the repair process (i.e., through incision) are performed (Dresler & Lieberman, 1983b). Therefore, isolated DNA was incubated with *E. coli* DNA polymerase I, all four nucleotides, and a trace amount of [^{3}H]dCTP to fill in the gaps left at the nascent repair sites. The ratio of ^{3}H/^{14}C for DNA from cells with BiodUMP-tagged repair patches was determined and compared with the ^{3}H/^{14}C ratio of the DNA from irradiated cells incubated without dNTPs during repair. Incorporation of ^{3}H by DNA polymerase I into the DNA of cells incubated with BiodUTP was about 23% of the level for DNA that was only incised (Table III). Furthermore, the fraction of ^{3}H incorporated by DNA polymerase I did not change for cells incubated an additional 24 h (in chase medium). Therefore, 75–80% of the repair patches could not be extended by DNA polymerase I, and (presumably) were ligated after a 30-min repair period and following a 24-h chase period. Since exonuclease III digests gave similar results (Figure 4B), it appears that 20–25% of the nascent repair patches containing BiodUMP are not efficiently ligated following repair synthesis.

Relative Nuclease Sensitivity of Repair-Incorporated BiodUMP. It has been shown using intact cells that repair-incorporated nucleotides are initially highly sensitive to staphylococcal nuclease but with increasing chase times these nucleotides become progressively more nuclease-resistant [reviewed in Smerdon (1989)]. Associated with this enhanced nuclease sensitivity are low levels of repair-incorporated nucleotides present in the nucleosomal core DNA, while the loss of nuclease sensitivity corresponds to a more random distribution of these nucleotides between nucleosomal core and linker regions (Smerdon & Lieberman, 1978b). This has been proposed to reflect an unfolding (or induced sliding) of nucleosomes during repair, and a refolding of repaired DNA after ligation (Smerdon, 1989). We analyzed the nuclease digestion characteristics of chromatin that had been repair-labeled with BiodUTP and [^{3}H]dCTP simultaneously for 30 min, and from cells in which these labels were chased for 0 or 24 h. Nuclei were digested with staphylococcal nuclease for various times, and the ratio of the fraction of the acid-soluble repair label

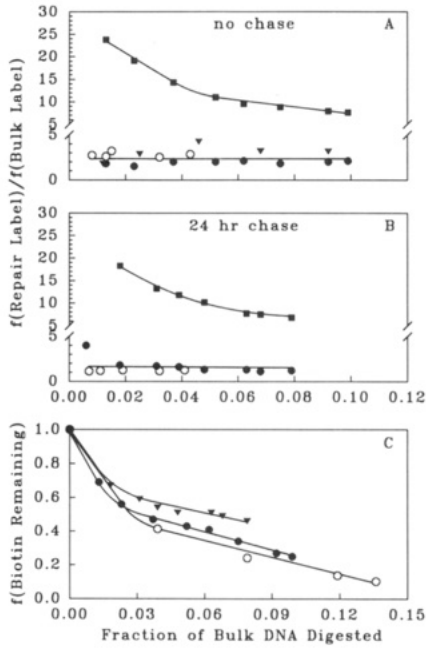

FIGURE 5: Staphylococcal nuclease sensitivity of repair-incorporated nucleotides. Normal human fibroblasts prelabeled with [^{14}C]dThd were irradiated with 12 J/m^2 UV light, permeabilized with 80 μg/mL lysolecithin, incubated at 37 °C in the presence of 3 μM BiodUTP and 20 μCi/mL [^{3}H]dCTP (16 Ci/mmol) for 30 min, and incubated in chase medium for 0 (A) or 24 h (B). Some cells were labeled with [^{3}H]dTTP (▼; Lorenz et al., 1988) instead of both BiodUTP (■) and [^{3}H]dCTP (●). Other cells were not permeabilized and labeled with [^{3}H]dThd (○). Nuclei were isolated and digested with staphylococcal nuclease (Materials and Methods). The ratio of the fraction of acid-soluble repaired DNA and acid-soluble bulk DNA is plotted versus the fraction of bulk DNA label that was acid-soluble (A and B). In panel C, values are shown for naked DNA from cells repair-labeled with BiodUTP (○) for 30 min and digested with staphylococcal nuclease. These values were determined from the disappearance of the signal on slot blots (e.g., Figure 1). For comparison, the values for BiodUMP from staphylococcal nuclease digested nuclei [no chase (●), 24-h chase (▼)] are included.

(^{3}H) and bulk DNA label (^{14}C) was plotted versus the fraction of the acid-soluble bulk DNA label. In these double-labeled nuclei, the rate of release of ^{3}H label was more rapid than the release of ^{14}C label after a 30-min repair period (approximately twice as fast), and the ratio of $f(^3\text{H})/f(^{14}\text{C})$ approached 1.0 after a 24-h chase time (Figure 5A,B). Double-labeled cells were used in order to compare the data obtained for intact cells, repair-labeled with [^{3}H]dThd, and lysolecithin-permeabilized cells, repair-labeled with [^{3}H]dTTP (Lorenz et al., 1988). All show similar results (Figure 5A,B). However, the biotin label was much more nuclease-sensitive than the radioactive repair label, immediately after the 30-min repair period, or following a 24-h chase period (Figure 5A,B). One explanation for these results is that staphylococcal nuclease may *preferentially* remove the biotinylated nucleotide. To test this possibility, naked DNA that was repair-labeled with BiodUTP was digested with staphyloccal nuclease, and indeed, the biotin label was selectively cleaved (Figure 5C).

Since the nuclease digestion characteristics of repair-incorporated nucleotides reflect nucleosome rearrangement during repair (Smerdon, 1989), it is clear from this work that staphylococcal nuclease cannot be used to probe this aspect of the repair process when BiodUMP is the repair label. Therefore, in order to determine if biotin-labeled repair patches become folded into nucleosomes, we first examined the protected DNA pattern on gels from nuclei repair-labeled with BiodUMP, and digested with the restriction enzymes *Hae*III and *Rsa*I. DNA from a staphylococcal nuclease digestion of

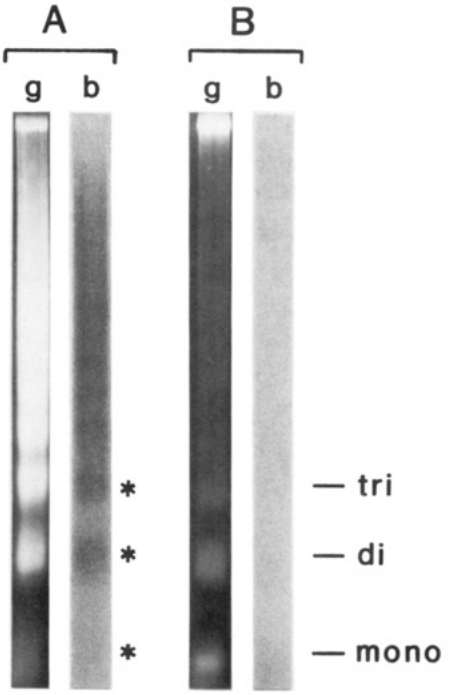

FIGURE 6: Restriction endonuclease digestion of DNA repair-labeled with BiodUTP. Nuclei from AG1518 cells, repair-labeled for 2 h with BiodUTP, were digested with *Hae*III and *Rsa*I (A) or with staphylococcal nuclease (B). Electrophoresis of the isolated DNA was carried out on a 1.5% agarose gel (g), and the biotin was detected on a Southern blot (b) as described under Materials and Methods. The stars (★) mark the position of the mono-, di-, and trinucleosome DNA.

the same nuclei was also run on the agarose gel as a control. Figure 6A shows that the biotin label is clearly visible in DNA following digestions with restriction enzymes. On the other hand, Figure 6B shows that the biotin label is barely detectable in the core DNA from staphylococcal nuclease digested nuclei, again demonstrating that BiodUMP is cleaved preferentially.

Restriction enzymes preferentially cleave restriction sites that are located in the linker regions of chromatin (e.g., Figure 6A). Therefore, mononucleosomal DNA also contains DNA fragments that are longer than core-length DNA (i.e., core DNA with parts of linker regions). Therefore, it is possible that the biotin label is incorporated *only* into linker DNA and is not associated with nucleosome core regions. To ascertain whether BiodUTP does become associated with this nucleosome subdomain, nuclei from AG1518 cells, which were repair-labeled with BiodUTP for 30 min and exposed to chase periods of 0 or 24 h, were digested with DNase I, and the DNA was subjected to electrophoresis on denaturing polyacrylamide gels. The DNA was electroblotted onto a nylon membrane, and the biotin was visualized as before. DNA from nuclei digested with DNase I yields an ~10.4-base repeat pattern on denaturing gels [e.g., see Smerdon and Lieberman (1980)]. Figure 7 shows that biotin was detected in fragments of DNA less than 146 bp long on Southern blots, both in the 0-h and in the 24-h chase samples, but not in DNA from unirradiated cells. It should be noted that the mass of BiodUMP is about double that of dTMP and DNA substituted with BiodUMP migrates more slowly in agarose gels than DNA without the biotinylated nucleotide (Langer et al., 1981). This explains the slight retardation in the fragments containing biodUMP on these gels (Figure 7). The results of these experiments clearly demonstrate that a portion of the repair-incorporated BiodUMP becomes associated with nucleosome core domains after insertion by repair enzymes.

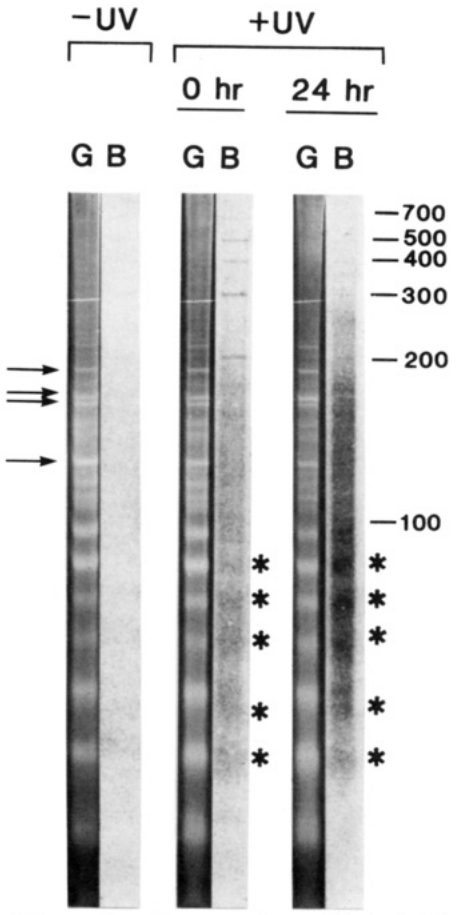

FIGURE 7: Denaturing gel patterns for nuclei repair-labeled with BiodUTP and digested with DNase I. AG1518 cells were irradiated (+UV) or mock-irradiated (−UV), with 12 J/m^2 UV light, permeabilized with lysolecithin, and incubated in repair mix containing BiodUTP for 30 min, and the label was chased for 0 or 24 h. Nuclei were digested with DNase I, and the DNA was electrophoresed on a denaturing polyacrylamide gel (G) and electroblotted onto a nylon membrane (B). The arrows (→) show small amounts of RNA that were present in the samples. The stars (★) mark the positions of some of the bands on the membrane that contain biotin.

Discussion

We have examined the effects of substituting BiodUTP for dTTP during repair synthesis in UV-irradiated, confluent human fibroblasts reversibly permeabilized with lysolecithin. Under these conditions, ~20% of the cells remain permeable to dNTPs and trypan blue for 2–3 h, while the remaining cells reseal within 30 min (Lorenz et al., 1988). It is likely that this same subpopulation of cells is labeled most effectively by BiodUTP. At present, we do not know how representative these cells are of the total cell population. However, our data strongly suggest that incorporation of BiodUTP occurs via excision repair synthesis in these cells. Incorporation of BiodUTP into two normal human fibroblast cell strains (AG1518 and IMR 90) is UV dose dependent, whereas xeroderma pigmentosum human cells (complementation group A) incorporate little (or no) BiodUTP after any of the UV doses used (Figure 1B). Furthermore, when these cells are labeled with [α-^{35}S]dCTP and BrdUTP under the same conditions, all of the label incorporated in the UV-irradiated cells bands with "light density" DNA in alkaline CsCl gradients (Lorenz et al., 1988; Lorenz, 1988), indicating that no residual DNA replicative synthesis takes place in the permeabilized confluent cells. These results indicate that BiodUTP is inserted into excision repair patches and that the projecting biotin side chain does not interfere significantly with repair synthesis.

The stability of the biotin tag in newly repaired DNA is of great importance for studies on repair sites long after the repair event. Hiriyanna et al. (1988) demonstrated that when heavily biotinylated circular DNA is microinjected into unfertilized *Xenopus laevis* eggs and embryos, it is stable for at least 4 h after microinjection. Our results indicate that in reversibly permeabilized confluent human fibroblasts, the biotin tag is not removed from previously repaired DNA over a 48-h period and only disappears slowly after longer incubation times (Figure 3). Furthermore, a second dose of UV radiation (to induce another round of repair) does not result in biotin removal (Table II). Possible explanations for these results are as follows: (1) The repair enzymes do not recognize BiodUMP as a damaged base. (2) The repair enzymes are unable to excise this nucleotide. (3) Excision repair is "processive", and the enzymes do not "go back over" already repaired DNA. However, when cells are stimulated to divide 72 h after UV irradiation, the amount of biotin is significantly reduced (Figure 3). This indicates that biotinylated nucleotides are eliminated during replication. Interestingly, Hiriyanna et al. (1988) observed that biotinylated circular plasmid DNA does undergo efficient replication in *Xenopus laevis* cells. Furthermore, Wauthier et al. (1990) demonstrated that DNA containing psoralen monoadducts is replicated with high efficiency in Chinese hamster ovary cells but these lesions effectively block transcription. Therefore, it is possible that some of the reduction in biotin that we observe after long chase times is due to a low level of replication. However, the majority of biotin lost after cell passage must reflect either increased cell death or removal of BiodUMP at the replication forks.

We also investigated the efficiency of ligation of biotinylated repair patches in reversibly permeabilized human cells. Using two different assays, we found that the majority of biotin-tagged repair patches (~80%) are efficiently ligated after the labeling period, albeit to a somewhat lower extent than those labeled with [^{3}H]dTTP (Figure 4). A similar difference was observed by Hunting et al. (1985a), who compared ligation of DNA repair patches, tagged with either dTTP or BiodUTP, in DNA from mechanically disrupted cells. The small difference between the extent of ligation of [^{3}H]dCTP- and BiodUTP-labeled repair patches that we observe could be due to the different techniques employed for detecting the sensitivity to exonuclease III (i.e., radioactive label released into acid-soluble form versus biotinylated label remaining on nylon filters). Since exonuclease III may be blocked at some biotinylated sites, we also used DNA polymerase I to extend nicks or gaps (with a free 3′-OH) at unligated repair patches (Caron et al., 1985). This assay gave a similar fraction (23%) of unligated repair sites as observed with the exonuclease III assay (18–30%). Also, with both assays, we did not observe any further ligation after a 24-h chase period, in contrast to reactions where dTTP was the repair label (Figure 4). Our results with [^{3}H]dTMP-labeled repair patches are in agreement with Lorenz et al. (1988) and indicate that the lack of ligation of 20–25% of the biotin-tagged repair patches is not the result of the permeabilization repair-labeling scheme. It is possible that in this subpopulation of repair patches the biotin label is located at (or near) the 3′ end, and ligation is inhibited.

Finally, we examined the chromatin refolding of repair patches containing BiodUMP and [^{3}H]dCMP. Staphylococcal nuclease sensitivity of repair-incorporated nucleotides has been used extensively to monitor transient changes in the nucleosome structure of nascent repair patches [reviewed in Smerdon (1989)]. We found that the [^{3}H]dCMP was more nuclease sensitive than the bulk DNA after a 30-min repair period, and

lost this enhanced sensitivity after a 24-h chase period, in agreement with previous findings for intact cells [e.g., see Smerdon et al. (1979)], lysolecithin-permeabilized cells (Lorenz et al., 1988), and mechanically disrupted cells (Hunting et al., 1985a). However, we found that BiodUMP is digested much more rapidly by staphylococcal nuclease both in nuclei and in naked DNA (Figure 5C). One possibility is that regions of biotinylated DNA are less stable in solution and these regions exhibit transient single-strandedness. Indeed, Langer et al. (1981) observed a decrease in the melting temperature of DNA heavily substituted with BiodUMP. It has long been known that staphylococcal nuclease not only has a preference for "A-T rich" regions in DNA (Anfinsen et al., 1971) but also degrades single-stranded DNA considerably faster (Marmur & Doty, 1959). Thus, local denaturation of biotin-tagged DNA could account for the high sensitivity of these regions to staphylococcal nuclease. Therefore, we examined nucleosome rearrangement in biotinylated repair patches using restriction enzymes and DNase I. Biotin was detected in mononucleosome DNA from restriction enzyme digests (Figure 6) and in submonomer fragments of DNase I digests (Figure 7), which result from digestion of DNA associated with nucleosome cores in nuclei (Lutter, 1979). These results indicate that biotin-tagged repair patches can indeed be folded into nucleosome cores.

In conclusion, we have demonstrated that BiodUTP is a suitable substitute for dTTP to study excision repair in lysolecithin-permeabilized human fibroblasts. The stability of this nucleotide analogue in the DNA of nonreplicating human cells makes it possible to study effects associated with excision repair (e.g., histone modifications) at long times after the repair event. Furthermore, the strong interaction between streptavidin and biotin should allow isolation of both newly repaired biotinylated DNA (Hunting et al., 1985a) and chromatin complexes (Vincenz et al., 1991).

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